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ARTICLE

# SIX4 promotes hepatocellular carcinoma metastasis through upregulating YAP1 and c-MET

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## Abstract

Metastasis is the main reason for high mortality in hepatocellular carcinoma (HCC) patients and the molecular mechanism remains unclear. Therefore, it is important to elucidate the mechanism underlying HCC metastasis. Here, we report a novel role of SIX homeobox 4 (SIX4), one of the SIX gene family, in promoting HCC metastasis. The elevated expression of SIX4 was positively correlated with loss of tumor encapsulation, microvascular invasion, higher TNM stage, and poor prognosis in human HCC. SIX4 expression was an independent and significant risk factor for the recurrence and survival in HCC patients. Upregulation of SIX4 promoted HCC invasion and metastasis, whereas downregulation of SIX4 decreased HCC invasion and metastasis. SIX4 transactivated Yes1 associated transcriptional regulator (YAP1) and MET proto-oncogene, receptor tyrosine kinase (MET) expression through directly binding to their promoters. Knockdown of YAP1 and c-MET inhibited SIX4-medicated HCC metastasis, while the stable overexpression of YAP1 and c-MET reversed the decreased metastasis induced by SIX4 knockdown. Hepatocyte growth factor (HGF), the specific ligand of c-MET, upregulated SIX4 expression through ERK/NF-κB pathway. Knockdown of SIX4 significantly decreased HGF-enhanced HCC metastasis. In human HCC tissues, SIX4 expression was positively correlated with nuclear YAP1, c-MET and HGF expression. Patients with positive coexpression of SIX4/ nuclear YAP1, SIX4/c-MET or HGF/SIX4 had the poorest prognosis. Moreover, the combination treatment of YAP1 inhibitor Verteporfin and c-MET inhibitor Capmatinib significantly suppressed SIX4-mediated HCC metastasis. In conclusion, SIX4 is a prognostic biomarker in HCC patients and targeting the HGF-SIX4-c-MET positive feedback loop may provide a promising strategy for the treatment of SIX4-driven HCC metastasis.

## Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death worldwide and most patients with HCC have limited treatment options [1, 2]. Although rational approaches have been achieved, the outcomes of HCC patients remain unsatisfactory. HCC is a highly invasive tumor with frequent intrahepatic and distant metastasis, which is the main reason for high recurrence and poor survival of HCC after surgical resection [3]. Thus, it is critical to discover the mechanism underlying HCC metastasis.

The Sine oculis homeobox (SIX) gene family, which belong to a superfamily of homeobox gene family, encode transcription factors containing two evolutionarily conserved domains, homeodomain (HD) and SIX domains. The HD domain is involved in DNA binding, whereas the SIX domain participates in protein-protein interaction [4]. SIX family members are initially identified as the key regulator of the tissue and organ development and construction

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during embryogenesis [4–6]. In humans, SIX family consists of six members, namely, SIX1, SIX2, SIX3, SIX4, SIX5, and SIX6. Accumulating evidence demonstrated that the deregulation of SIX family genes contribute to cancer initiation, progression, and metastasis [4]. Overexpression of SIX1 and SIX2 promotes cancer proliferation, invasion and metastasis, and indicates poor prognosis in several human cancers including colorectal cancer, breast cancer, gastric cancer, and lung cancer [7–12]. In contrast, SIX3 expression is dramatically decreased in many human cancers, and functions as a tumor suppressor gene to suppress proliferation, invasion, and metastasis [13, 14]. These studies indicate that deregulation of SIX family genes play critical roles in cancer progression and metastasis.

To investigate the role of *SIX* genes in HCC, we detected *SIX* genes expression in 10 normal liver tissues and 30 paired HCC tissues and adjacent nontumor tissues. Among the 6 *SIX* genes, SIX4 was the most upregulated genes (Supplementary Fig. S1). SIX4 contains 760 amino acids and localizes to the nucleus [4]. SIX4 functions as a transactivator and participates in organ development including myogenesis and neurogenesis [4]. Recent studies reported that elevated expression of SIX4 was associated with poor prognosis in breast cancer, lung cancer, and colorectal cancer, and SIX4 functioned as an oncogene by facilitating cancer cell proliferation and metastasis [15–17]. However, the expression and functional role of SIX4 in human HCC remains unknown.

Hepatocyte growth factor (HGF) binds to its receptor tyrosine kinase c-MET and activates mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways [18]. HGF/c-MET signaling regulates the epithelial-to-mesenchymal and cell migration during embryogenesis and promotes liver regeneration and skin repair [18]. Genetic mouse lacking c-MET or HGF are embryonic lethal [19]. HGF/c-MET signaling promotes liver repair through inducing hepatocyte proliferation, survival, regeneration, and suppress liver fibrosis [19, 20]. Recent studies reported that HGF/c-MET signaling play an important role in promoting HCC growth, angiogenesis, and metastasis [19–22]. HGF and c-MET transgenic mice are more likely to form hepatocellular adenomas and carcinomas [19]. The elevated expression of c-MET and HGF have been reported in human HCC tissues and indicates poor prognosis [19, 21]. Moreover, c-MET is identified as a critical therapeutic target in human HCC [20, 23]. These studies demonstrate the important role of HGF/c-MET signaling in HCC progression and metastasis. However, the exact mechanism of HGF/c-Met signaling in HCC metastasis is still largely unknown.

To date, no studies have reported the expression and potential function of SIX4 in HCC. In this study, we demonstrated that overexpression of SIX4 promoted HCC metastasis by upregulating Yes1 associated transcriptional regulator (YAP1) and c-MET expression. HGF upregulated SIX4 expression through the extracellular-signal-regulated kinase (ERK)/nuclear factor κB (NF-κB) pathway, which formed an HGF-SIX4-c-MET positive feedback loop. Furthermore, combination treatment of YAP1 inhibitor Verteporfin and c-MET inhibitor Capmatinib significantly suppressed SIX4-mediated HCC metastasis.

## Results

### SIX4 is significantly upregulated in HCC tissues and indicates poor prognosis in HCC patients

We first screened the expression profile of *SIX* genes in HCC tissues compared to that of adjacent nontumor tissues by Real-time PCR. The mRNA levels of *SIX1*, *SIX2 SIX4*, and *SIX5* were increased in HCC tissues than in adjacent nontumor tissues. Among them, *SIX4* exhibited the largest fold change. In contrast, the mRNA levels of *SIX3* were significantly decreased in HCC tissues. In addition, the mRNA level of *SIX6* showed no obvious change in HCC tissues compared with those in adjacent nontumor tissues (Supplementary Fig. S1).

To investigate the potential role of SIX4 in HCC, we analyzed its expression in a cohort of 50 paired HCC tissues. The mRNA levels of *SIX4* were dramatically upregulated in HCC tissues than in adjacent nontumorous tissues and normal liver tissues (Fig. 1a left). The *SIX4* mRNA expression was higher in patients with recurrence or metastasis than in patients without recurrence or metastasis (Fig. 1a middle). In addition, the mRNA levels of *SIX4* were compared in primary and metastatic HCCs in 30 pairs of HCC specimens. Real-time PCR analysis showed that *SIX4* mRNA expression was much higher in metastatic HCC tissues than in primary HCC tissues (Fig. 1a right). A representative case of immunohistochemical staining of SIX4 was shown in Fig. 1b. A higher protein level of SIX4 expression was observed in metastatic HCC samples than in primary HCC samples.

We next analyzed the protein expression and the clinical significance of SIX4 with a tissue array of 220 HCC patient samples (Cohort I, $n = 220$) using immunohistochemical (IHC) staining. SIX4 protein expression was significantly up-regulated in HCC tissues than in adjacent nontumorous tissues, and SIX4 expression was primarily localized to the nucleus (Fig. 1c). The IHC score of HCC tissues was higher than that of adjacent nontumor tissues (Fig. 1c, and Supplementary Fig. S2). Patients with positive SIX4 expression had shorter overall survival times and higher recurrence rates than patients with negative SIX4 expression (Fig. 1d). The elevated expression of SIX4 was significantly

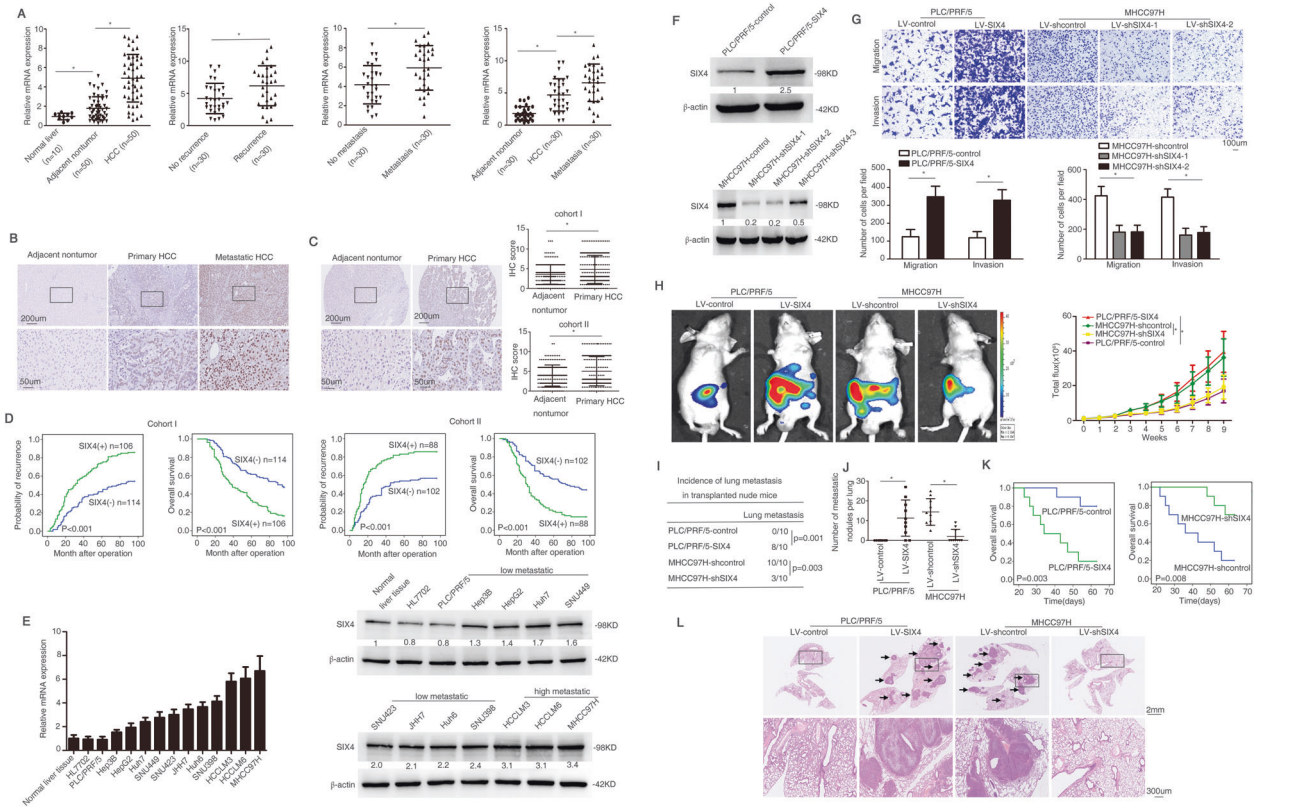


**Fig. 1 Overexpression of SIX4 promotes HCC metastasis and indicates poor prognosis in HCC patients. a** Relative SIX4 mRNA expression in 10 normal liver tissues and 50 paired HCC and adjacent nontumorous tissues. Relative SIX4 mRNA expression in HCC patients with or without recurrence ($n = 30$). Relative SIX4 mRNA expression in HCC patients with or without metastasis ($n = 30$). Relative SIX4 mRNA expression in HCC patients with or without metastasis ($n = 30$) and in adjacent nontumorous tissues ($n = 30$). **b** Representative image of SIX4 expression using IHC staining in adjacent nontumorous tissues, HCC tissues and metastatic HCC tissues. **c** Representative image of the IHC staining and IHC scores of SIX4 in two HCC cohorts. Statistical analysis was performed by chi-squared test. **d** Kaplan–Meier analysis of the association of SIX4 expression and recurrence or overall survival in two cohorts of HCC. **e** Relative mRNA and protein expression of SIX4 in normal liver tissue and HCC cell lines. **f** Western blot analysis was used to show SIX4 expression in PLC/PRF/5 and MHCC97H cells after lentivirus transfection. **g** Transwell shown the migratory and invasive abilities of HCC cells after the changs of SIX4 expression. **h–l** In vivo assays shown that SIX4 knockdown can inhibit HCC metastasis. **h** The nude mice were injected with the indicated cells in the liver. Bioluminescent images were shown. **i** Incidence of lung metastasis in the treated nude mice. **j** The number of metastatic nodules in lung. **k** Overall survival time of nude mice in different groups was shown. **l** Representative HE staining images of lung tissues from the different groups were shown. $^*P < 0.05$.

correlated with loss of tumor encapsulation, microvascular invasion, and a higher tumor-nodule-metastasis (TNM) stage (Table 1). Multivariate analysis showed that SIX4 expression was an independent and significant risk factor for recurrence and reduced survival (Supplementary Table S1). The expression and clinical significance of SIX4 was validated in an independent cohort of HCC tissues (Cohort II, $n = 190$). Similarly, overexpression of SIX4 was significantly correlated with loss of tumor encapsulation, microvascular invasion, higher TNM stage (Table 1), and indicated poor prognosis (Fig. 1d). Multivariate analysis showed that SIX4 over-expression was an independent predictor for postoperative recurrence and overall survival (Supplementary Table S1). Taken together, these studies suggested that SIX4 was a prognostic biomarker in HCC patients.

## Overexpression of SIX4 promotes HCC invasion and metastasis

We then detected SIX4 expression in HCC cell lines and found that SIX4 expression was higher in HCC cells with high metastatic capability than in HCC cells with low metastatic capability (Fig. 1e). PLC/PRF/5 and MHCC97H cells were used to establish stable cell lines, PLC/PRF/5-SIX4, MHCC97H-shSIX4-1 and MHCC97H-shSIX4-2, with lentivirus infection (Fig. 1f). Overexpression of SIX4 increased the migrative and invasive ability of PLC/PRF/5 cells, while the knockdown of SIX4 decreased the migratory and invasive ability of MHCC97H cells (Fig. 1g). The in vivo metastatic assay showed that overexpression of SIX4 expedited the lung metastasis rate and increased the number of metastatic lung nodules and lowered the survival

**Table 1** Correlation between SIX4 expression and clinicopathological characteristics of HCCs in two independent cohorts of human HCC tissues.

| Clinicopathological variables | Cohort I | | | Cohort II | | |
|---|---|---|---|---|---|---|
| | Tumor SIX4 expression | | *P* value | Tumor SIX4 expression | | *P* value |
| | Negative ($n = 114$) | Positive ($n = 106$) | | Negative ($n = 102$) | Positive ($n = 88$) | |
| Age | 52.86 (11.641) | 50.87 (12.884) | 0.192 | 51.01 (8.388) | 50.82 (7.848) | 0.307 |
| Sex | | | | | | |
| Female | 25 | 15 | 0.163 | 15 | 16 | 0.559 |
| Male | 89 | 91 | | 87 | 72 | |
| Serum AFP | | | | | | |
| ≤20 ng/ml | 26 | 23 | 0.872 | 22 | 21 | 0.731 |
| >20 ng/ml | 88 | 83 | | 80 | 67 | |
| Virus infection | | | | | | |
| HBV | 85 | 73 | 0.784 | 76 | 65 | 0.583 |
| HCV | 11 | 14 | | 8 | 11 | |
| HBV + HCV | 5 | 6 | | 4 | 4 | |
| None | 13 | 13 | | 14 | 8 | |
| Cirrrhosis | | | | | | |
| Absent | 28 | 31 | 0.450 | 24 | 18 | 0.726 |
| Present | 86 | 75 | | 78 | 70 | |
| Child-pugh score | | | | | | |
| Class A | 87 | 78 | 0.644 | 89 | 69 | 0.122 |
| Class B | 27 | 28 | | 13 | 19 | |
| Tumor number | | | | | | |
| Single | 77 | 44 | <0.001 | 62 | 52 | 0.882 |
| Multiple | 37 | 62 | | 40 | 36 | |
| Maximal tumor size | | | | | | |
| ≤5 cm | 40 | 43 | 0.408 | 67 | 40 | 0.006 |
| >5 cm | 74 | 63 | | 35 | 48 | |
| Tumor encapsulation | | | | | | |
| Absent | 37 | 62 | <0.001 | 23 | 42 | <0.001 |
| Present | 77 | 44 | | 79 | 46 | |
| Microvascular invasion | | | | | | |
| Absent | 79 | 42 | <0.001 | 73 | 30 | <0.001 |
| Present | 35 | 64 | | 29 | 58 | |
| Tumor differentiation | | | | | | |
| I–II | 98 | 73 | 0.003 | 87 | 46 | <0.001 |
| III–IV | 16 | 33 | | 15 | 42 | |
| TNM stage | | | | | | |
| I–II | 94 | 60 | <0.001 | 87 | 50 | <0.001 |
| III | 20 | 46 | | 15 | 38 | |

time of the nude mice. In contrast, downregulation of SIX4 expression largely impaired the lung metastasis rate and the number of metastatic lung nodules and prolonged the survival time of the nude mice (Fig. 1h–l). These studies suggested that SIX4 promoted HCC invasion and metastasis.

We detected the effect of SIX4 on HCC proliferation and tumor growth. Cell Counting Kit-8 (CCK8) and colony formation assays showed that overexpression of SIX4 increased cell proliferation of PLC/PRF/5 cells, whereas knockdown of SIX4 decreased cell proliferation of MHCC97H cells (Supplementary Fig. S3A, B). In vivo tumorigenicity assays showed that overexpression of SIX4 increased tumor growth of PLC/PRF/5 cells, whereas knockdown of SIX4 decreased tumor growth of MHCC97H cells, which was further confirmed by the IHC staining for

Ki67 in the xenograft tumors of different groups (Supplementary Fig. S3C–E). These studies suggested that SIX4 promoted HCC cell proliferation.

## *YAP1* and *MET* are two direct transcriptional targets of SIX4

To investigate the molecular mechanism underlying SIX4-mediated HCC metastasis, we compared the mRNA expression profiles between PLC/PRF/5-SIX4 and PLC/PRF/5-control cells using a Liver cancer RT$^2$ Profiler PCR Array. Overexpression of SIX4 induced the expression of several liver cancer-related genes, including *MET*, *YAP1*, *TLR4*, *EGFR*, *HGF*, *MYC*, *CTNNB1*, *CXCR4*, and *ADAM17* (Supplementary Table S2). Among these genes, both c-MET and YAP1 have been reported to be dramatically upregulated in HCC tissues and indicated poor prognosis [19, 24]. Overexpression of either c-MET or YAP1 promoted HCC invasion and metastasis. Considering the critical role of c-MET and YAP1 in metastasis, we determined whether they were involved in SIX4-mediated HCC metastasis. Overexpression of SIX4 upregulated YAP1 and c-MET expression, whereas knockdown of SIX4 reduced YAP1 and c-MET expression (Fig. 2a). Luciferase reporter assay showed that overexpression of SIX4 transactivated the *YAP1* and *MET* promoter activities (Fig. 2b).

In order to test how SIX4 regulated *YAP1* and *MET* expression, the promoter sequences of *YAP1* and *MET* were analyzed and two putative SIX4 binding motifs were found in the *YAP1* and *MET* promoters respectively. A series of reporter plasmid constructs containing truncated or mutated *YAP1* and *MET* promoter sequences were designed. We found that the deletion of the region between −940 and −238 bp significantly reduced the *YAP1* reporter activity medicated by SIX4 overexpression and the mutation of putative binding site 1 in the *YAP1* promoter reduced the activity of the luciferase reporter that was mediated by SIX4 overexpression (Fig. 2c). Similarly, deletion of the region between −1427 and −504 bp decreased the reporter activity of *MET* and the mutation of putative binding site 1 in the *MET* promoter reduced the activity of the luciferase reporter induced by SIX4 overexpression (Fig. 2d). Moreover, chromatin immunoprecipitation (ChIP) assays showed that SIX4 directly bound to the *YAP1* and *MET* promoters in PLC/PRF/5-SIX4 cells lines and human HCC samples (Fig. 2e, f). These findings demonstrated that *YAP1* and *MET* were direct transcriptional targets of SIX4.

Nuclear YAP1, which represents the activated status of YAP1, plays an important role in HCC progression [25]. Overexpression of SIX4 increased the expression of nuclear YAP1, whereas knockdown of SIX4 decreased the expression of nuclear YAP1 (Fig. 2g). Furthermore, overexpression of SIX4 increased the expression of YAP1 target genes, such as CTGF, CYR61, JAG1 and c-MYC, whereas knockdown of SIX4 decreased YAP1 target genes' expression (Fig. 2h).

c-MET is predominantly regulated by its phosphorylation [20]. Several studies reported that overexpression of c-MET causes receptor dimerization and activation and c-MET can be auto-phosphorylated in MHCC97H cells [20, 26, 27]. To determine whether SIX4-mediated c-MET overexpression regulates c-MET phosphorylation and the activation of downstream signaling pathway, we detected the phosphorylation of c-MET and the downstream signaling pathway of c-MET. Western blotting analysis showed that overexpression of SIX4 increased the phosphorylation of c-MET and activated downstream signaling pathway, such as ERK1/2, JNK, p38, AKT, and NF-κB pathway (Fig. 2i).

## SIX4 promotes HCC invasion and metastasis through upregulating YAP1 and c-MET

To explore whether YAP1 and c-MET were involved in SIX4-medicated HCC metastasis, we knocked down the expression of YAP1 and c-MET in PLC/PRF/5-SIX4 cells and ectopically overexpressed YAP1 and c-MET in MHCC97H-shSIX4 cells with lentivirus transfection (Fig. 3a). Transwell assay showed that knockdown of YAP1 and c-MET significantly decreased SIX4-enhanced migratory and invasive abilities, whereas overexpression of YAP1 and c-MET rescued the reduced migratory and invasive abilities induced by SIX4 knockdown (Fig. 3b). The in vivo metastatic assay showed that knockdown of YAP1 and c-MET lowered the incidence of lung metastasis and the number of metastatic lung nodules and prolonged the overall survival of the PLC/PRF/5-SIX4 group (Fig. 3c–g). In contrast, overexpression of YAP1 and c-MET reversed decreased lung metastasis and the number of metastatic lung nodules in MHCC97H-shSIX4 group and decreased the overall survival of this group (Fig. 3c–g). These results indicated that SIX4 promoted HCC metastasis by upregulating YAP1 and c-MET expression.

## HGF upregulates SIX4 expression through the c-MET/ERK/NF-kB signaling pathway

Since c-MET was involved in SIX4-mediated HCC metastasis, its specific ligand HGF attracted our attention. HGF-c-MET signaling plays a critical role in promoting HCC metastasis [28]. HGF was identified as a driver oncogene and elevated expression of HGF was positively correlated with poor prognosis in human HCC [21]. Considering the important roles of both HGF and SIX4 in HCC metastasis, we determined whether HGF regulates SIX4 expression. To test this hypothesis, PLC/PRF/5 cells with low endogenous SIX4 expression were treated with HGF.

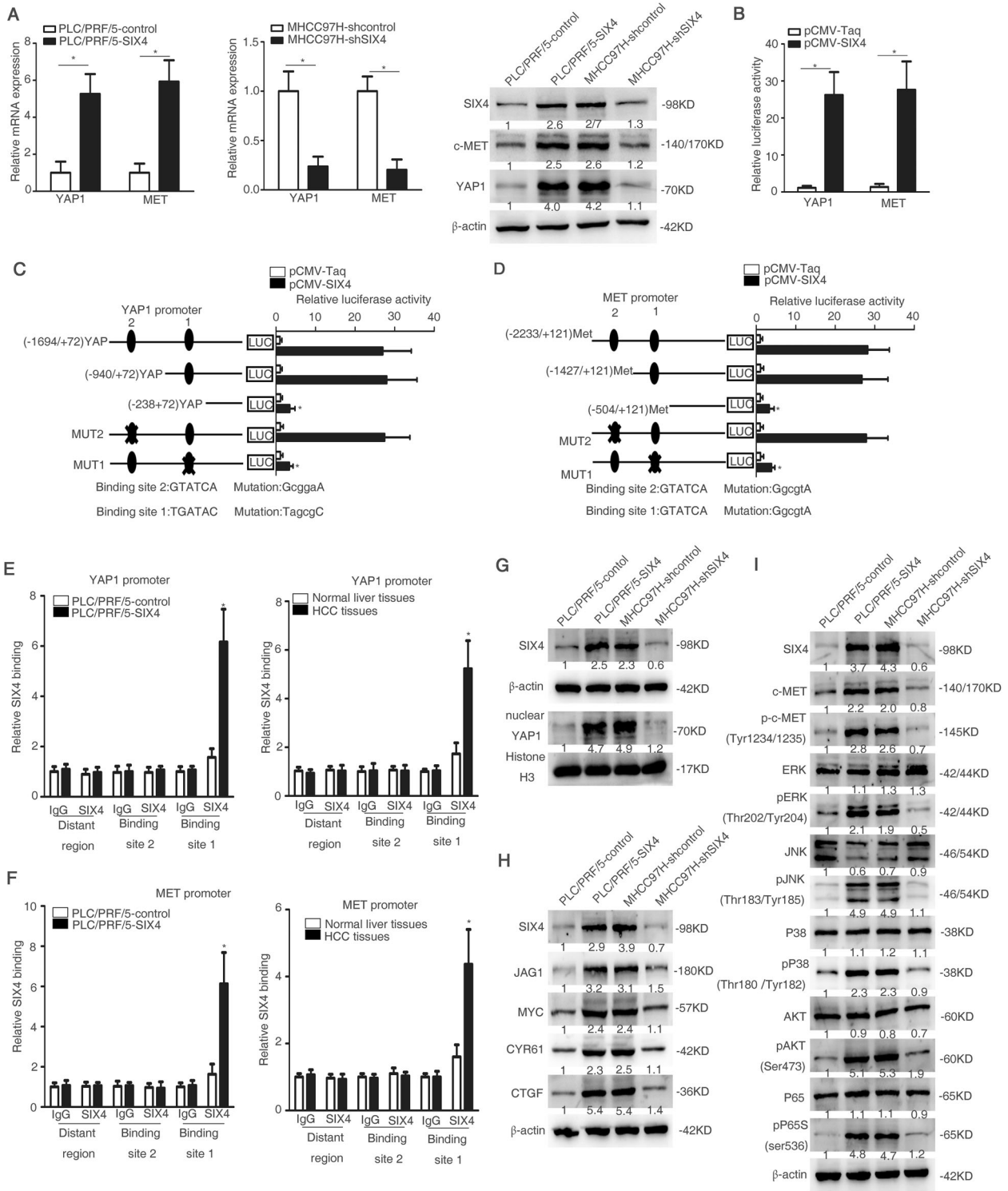
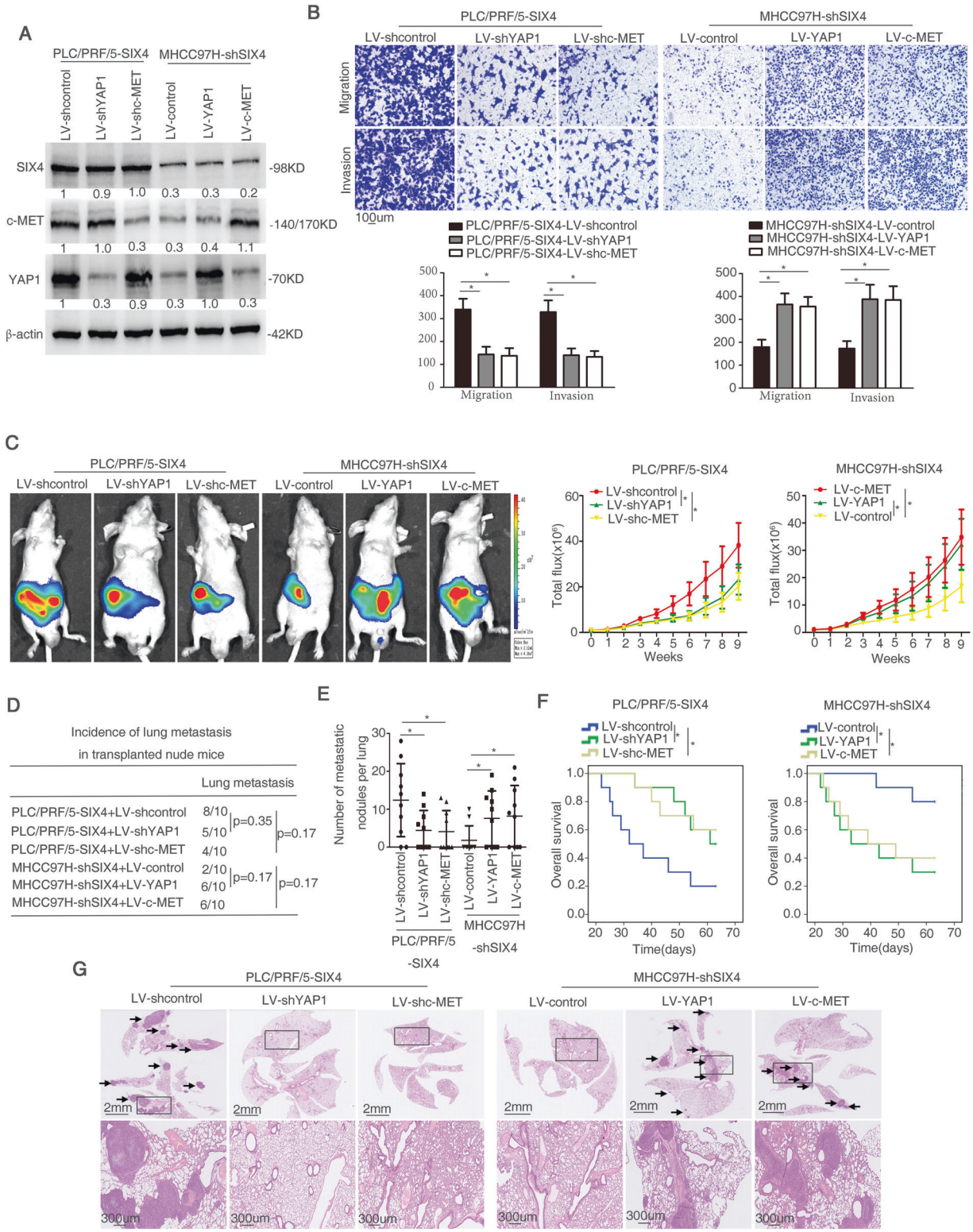



**Fig. 2 YAP1 and MET are two direct transcriptional targets of SIX4. a** Relative mRNA expression and protein levels of YAP1 and c-MET were analyzed by RT-qPCR and western blotting in the indicated cells. **b** Luciferase reporter assay was performed in the indicated cells cotransfected with pCMV-SIX4 and the *YAP1* or *MET* promoter luciferase construct. **c**, **d** Serially truncated and mutated *YAP1* or *MET* promoter constructs were cotransfected with pCMV-SIX4 in PLC/PRF/5 cells and relative luciferase activities were measured. **e**, **f** ChIP assays revealed the binding enrichment of SIX4 in *YAP1* or *MET* promoters in HCC cell lines and in HCC specimens. **g** Western blotting analysis of the expression of nuclear YAP1 in the indicated HCC cells. **h** Western blotting analysis of the expression of YAP1 target genes JAG1, MYC, CYR61 and CTGF in the indicated HCC cells. **i** Western blotting analysis of the expression of p-c-MET and downstream signaling pathway in the indicated HCC cells.




HGF treatment increased SIX4 expression in a dose-dependent manner (Fig. 4a). Of note, HGF treatment transactivated *SIX4* promoter activity (Fig. 4b).

To identify the cis-regulatory elements, which involved in HGF-induced SIX4 expression, the −1845 to +135 bp region and a series of truncations and mutations of the human *SIX4* promoter were generated. A significant reduction of *SIX4* promoter activity was observed when PLC/PRF/5 cells were transfected with the truncated (−365 to −43) SIX4 promoter construct, suggesting that this

◀ **Fig. 3 SIX4 promotes HCC invasion and metastasis through upregulating YAP1 and c-MET. a** Western blotting was used to show the SIX4, YAP1 and c-MET expression in HCC cells transfected with lentivirus. **b** Transwell shown the migratory and invasive abilities in PLC/PRF/5-SIX4 cells with YAP1 or c-MET knockdown and in MHCC97H-shSIX4 cell with YAP or c-MET overexpression. **c–g** In vivo assays shown that SIX4 promotes HCC metastasis through upregulating YAP1 and c-MET. **c** The nude mice were implanted with the indicated cells in the liver. Representative bioluminescent images in the different groups were shown. **d** Incidence of lung metastasis in the treated nude mice. **e** The number of lung metastatic nodules in the lung was counted. **f** Overall survival time of the treated nude mice in different groups was shown. **g** Representative HE staining images of lung tissues from different groups were shown. $*P < 0.05$.

region was crucial for HGF-induced *SIX4* promoter transactivation. Two potential NF-kB binding sites located in this region. Site-directed mutagenesis showed that mutation of second binding site significantly reduced the *SIX4* promoter activity induced by HGF (Fig. 4c). Knockdown of p65 which is a key subunit of NF-kB significantly impaired HGF-induced SIX4 overexpression and *SIX4* promoter transactivation (Fig. 4d). Similarly, an NF-kB inhibitor treatment (BAY11-7082) significantly inhibited HGF-mediated *SIX4* promoter transactivation and SIX4 upregulation (Fig. 4e).

HGF has been reported to activate MAPK and PI3K-AKT pathways [18]. In order to test which pathway was involved in HGF-mediated SIX4 overexpression, PLC/PRF/5 cells were treated with ERK, c-Jun-N-terminal kinase (JNK), p38 kinases or PI3K inhibitors. Pretreatment of cells with the ERK inhibitor significantly inhibited HGF-mediated SIX4 overexpression, whereas pretreatment of cells with the JNK, P38 or PI3K inhibitors had no significant effect (Fig. 4f). Furthermore, a ChIP assay demonstrated that the ERK inhibitor treatment inhibited the binding of NF-kB to the *SIX4* promoter, while the JNK, P38 or PI3K inhibitor treatment showed no effect (Fig. 4g). These results suggested that HGF upregulated SIX4 expression through the ERK/NF-kB signaling pathway.

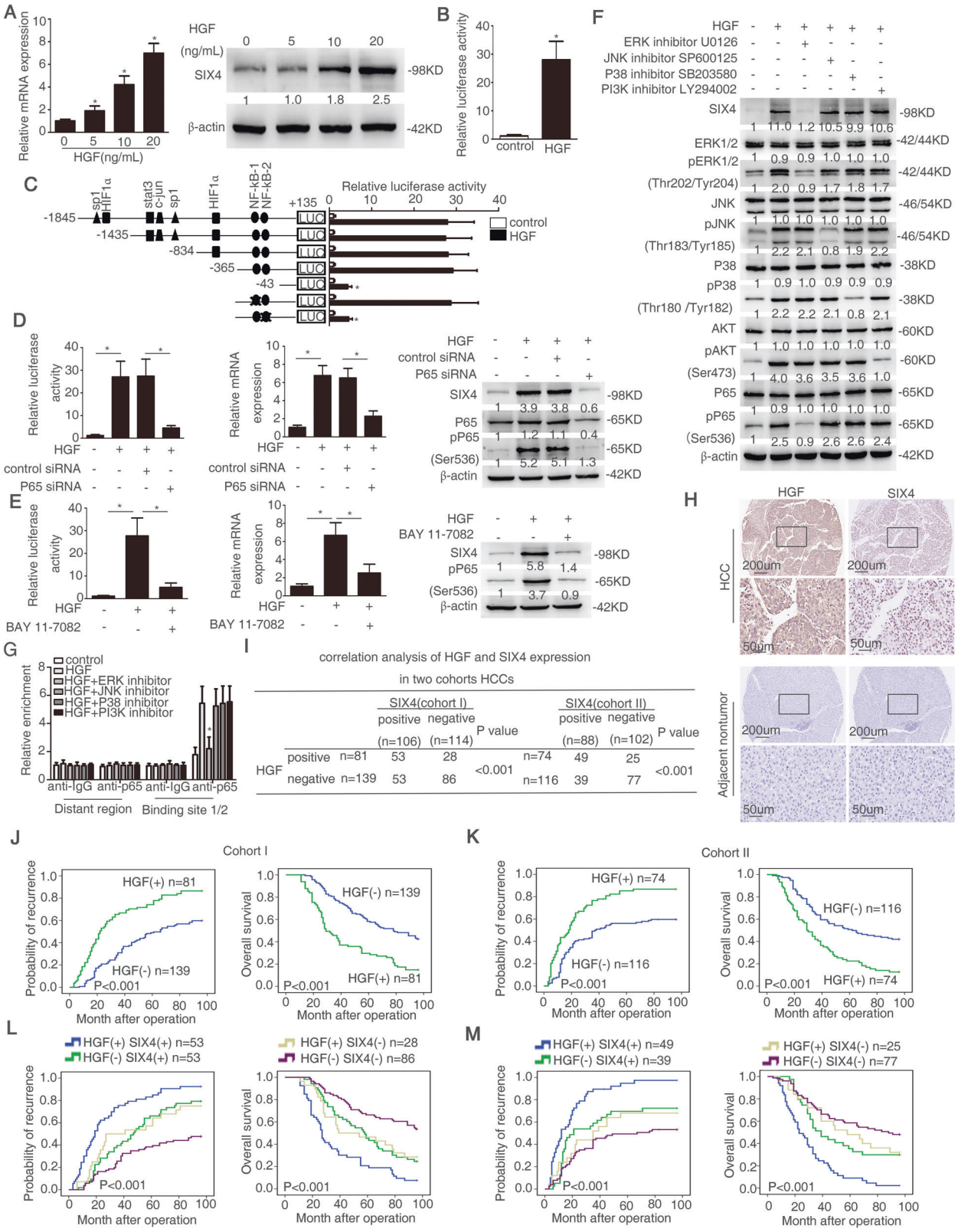
To confirm the clinical importance of HGF in HCC, IHC analysis was performed in two independent HCC cohorts. HGF expression was upregulated in HCC tissues than that in adjacent nontumor tissues (Fig. 4h). In both cohorts, overexpression of HGF was positively correlated with microvascular invasion, poorer tumor differentiation and higher TNM stage (Supplementary Table S3). SIX4 expression was positively correlated with HGF expression (Fig. 4i). Patients with positive expression of HGF had a higher recurrence rate and poorer overall survival time than patients with negative expression of HGF (Fig. 4j, k). Furthermore, Kaplan–Meier analysis exhibited that patients with positive coexpression of HGF/SIX4 suffered from the highest recurrence risk and shortest survival times in both cohorts (Fig. 4l, m). Moreover, we detected HGF expression in the metastatic lung nodules from PLC/PRF/5-SIX4 xenograft group, indicating that HGF expression was upregulated in the metastatic milieu in the tumor models when SIX4 was expressed (Supplementary Fig. S4).

## SIX4 is essential for HGF-induced HCC metastasis

As SIX4 was upregulated by HGF and promoted HCC metastasis, we determined whether SIX4 was involved in HGF-mediated HCC metastasis. PLC/PRF/5 cells were infected with Lentivirus LV-shSIX4 and then were treated with HGF (Fig. 5a). HGF treatment significantly increased the migratory and invasive abilities of PLC/PRF/5 cells, whereas knockdown of SIX4 largely lowered the increased migratory and invasive abilities induced by HGF (Fig. 5b). We then established a stable cell line PLC/PRF/5-HGF through lentiviral transduction and knocked down the expression of SIX4 in PLC/PRF/5-HGF cells (Fig. 5c). Stable overexpression of HGF significantly increased the migratory and invasive abilities of PLC/PRF/5 cells while SIX4 knockdown decreased the enhanced migratory and invasive abilities of PLC/PRF/5-HGF cells (Fig. 5d). The in vivo metastasis experiment showed that overexpression of HGF increased the incidence of lung metastasis and the number of metastatic lung nodules and decreased the overall survival in PLC/PRF/5-HGF group compared with that in control group (PLC/PRF/5-control). However, knockdown of SIX4 decreased the incidence of lung metastasis and the number of metastatic lung nodules while extended the overall survival in the PLC/PRF/5-HGF group (Fig. 5e–i). These results demonstrated that SIX4 was essential for HGF-induced HCC metastasis.

## SIX4 expression is positively correlated with nuclear YAP1 and c-MET expression in human HCC tissues

We further evaluated the possible association between SIX4 and nuclear YAP1 or c-MET in two independent cohorts of HCC patients. Representative images of the IHC staining were shown in Fig. 6a. IHC staining showed that YAP1 was mainly localized in the nucleus (Fig. 6a). SIX4 expression was positively correlated with nuclear YAP1 and c-MET expression in both cohorts (Fig. 6b, c). The elevated expression of both nuclear YAP1 and c-MET were positively correlated with microvascular invasion and higher TNM stage (Supplementary Tables S4, 5). Patients with positive expression of nuclear YAP1 or c-MET exhibited a higher recurrence rate and shorter overall survival compared with patients with negative expression of nuclear YAP1 or c-MET (Fig. 6d, e). Furthermore, Kaplan–Meier analysis exhibited that HCC patients with positive coexpression of either SIX4/ nuclear YAP1 or SIX4/c-MET had the highest recurrence risk and shortest survival time in both HCC cohorts (Fig. 6f, g).




Moreover, the mRNA and protein levels of YAP1 and c-MET were compared in primary and metastatic HCCs in 30 pairs of HCC specimens. Real-time PCR analysis showed that the mRNA expressions of both YAP1 and MET were much higher in metastatic HCC tissues than in primary HCC tissues (Supplementary Fig. S5A). Representative

◀ **Fig. 4 HGF upregulates SIX4 expression through ERK/NF-kB signaling pathway. a** Relative mRNA and protein level of SIX4 were analyzed in PLC/PRF/5 cells after treated with HGF. **b** SIX4 promoter luciferase activity was measured after HGF treatment in PLC/PRF/5 cells. **c** Serially truncated and mutated SIX4 promoter constructs were transfected into PLC/PRF/5 cells and treatment with HGF and relative luciferase activity were detected. **d**, **e** PLC/PRF/5 cells were transfected with p65 siRNA or control siRNA, control or NF-kB inhibitor BAY 11-7082 and then treatment with HGF. SIX4 promoter activity and expression were measured by luciferase activity assay, RT-qPCR and Western blotting. **f** PLC/PRF/5 cells were treated with inhibitor of ERK, JNK, P38 and PI3K and then stimulated with HGF. Western blotting was used to detect the expression of SIX4 as well as the total and phosphorylated levels of ERK, JNK, P38, AKT and P65. **g** A ChIP assay shown the relative enrichment of P65 on SIX4 promoter when the PLC/PRE/5 cells were treated with HGF and inhibitor of ERK, JNK, P38 or PI3K. **h** Representative IHC staining images of SIX4 and HGF in HCC samples. **i** The correlation analysis of the SIX4 and HGF expression in two independent cohorts of HCC patients. **j**, **k** Overall survival time and recurrence in HCC patients with positive or negative expression of HGF in cohort I (**j**) and cohort II (**k**) were shown. **l**, **m** Kaplan–Meier analyzed recurrence and overall survival times of patients with coexpression of HGF/SIX4 or HGF/SIX4 in cohort I (**l**) and cohort II (**m**). $^{*}P < 0.05$.

cases of immunohistochemical staining of YAP1 and c-MET were shown in Supplementary Fig. S5B, C. The protein levels of both YAP1 and MET were much higher in metastatic HCC tissues than in primary HCC tissues (Supplementary Fig. S5B, C).

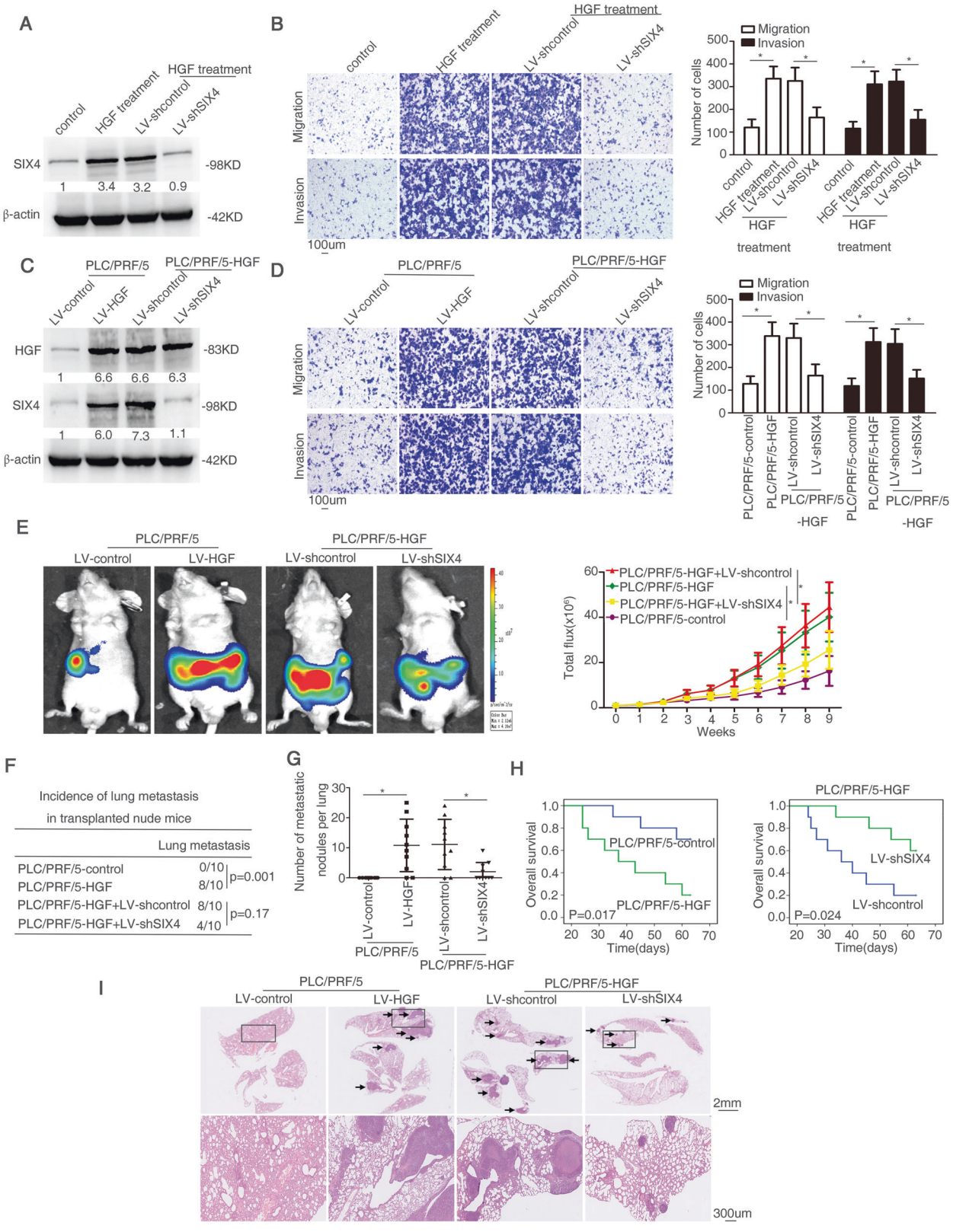
### Combined treatment of YAP1 and c-MET inhibitor significantly suppress SIX4-medicated HCC metastasis

Verteporfin is a YAP1 inhibitor, which disrupts YAP-TEAD interactions [29]. Capmatinib is a highly selective and potent c-Met inhibitor which inhibit c-MET activation [30]. Our above research has demonstrated that HGF/c-MET signaling induced SIX4 overexpression, and SIX4 promoted HCC metastasis through upregulating YAP1 and c-MET expression. Therefore, we determined whether combination treatment of YAP1 and c-MET inhibitors had any effect on SIX4-mediated HCC metastasis. PLC/PRF/5-SIX4 cells were treated with YAP1 inhibitor Verteporfin or c-MET inhibitor Capmatinib alone, or the combination of both inhibitors, and the protein levels of YAP1, c-MET and phospho-MET were shown (Fig. 7a). Treatment with Verteporfin or Capmatinib alone partially decreased the migratory and invasive abilities of PLC/PRF/5-SIX4 cells, whereas combination of the two agents dramatically lowered the migratory and invasive abilities of PLC/PRF/5-SIX4 cells (Fig. 7b). In order to further investigate this effect, we designed the in vivo experiment (Fig. 7c). The in vivo metastasis assay showed that Verteporfin or Capmatinib treatment alone partially decreased the incidence of lung metastasis and the number of metastatic lung nodules while partially increasing the overall survival time of the PLC/PRF/5-SIX4 group, whereas the combination of Verteporfin and Capmatinib dramatically inhibited the number of lung metastasis and largely prolonged survival time compared with control or single agent treatment (Fig. 7d–h). These studies suggested that the combination treatement of YAP1 inhibitor Verteporfin and c-MET inhibitor Capmatinib significantly suppressed SIX4-mediated HCC metastasis.

## Discussion

Metastasis is still the main reason for the high mortality of HCC patients [3]. Thus, exploring the metastatic mechanism and the potential therapeutic target is urgent needed. In this study, we found that SIX4 was upregulated in HCC tissues compared with adjacent nontumorous tissues. Overexpression of SIX4 was significantly correlated with loss of tumor encapsulation, microvascular invasion, and a higher TNM stage. HCC patients with positive SIX4 expression had shorter overall survival times and higher recurrence rates than patients with negative SIX4 expression. A multivariate analysis revealed that SIX4 expression was an independent risk factor for higher recurrence and shorter overall survival in HCC patients. Furthermore, SIX4 expression was higher in HCC tissues from patients who developed metastasis than in HCC tissues from patients who did not develop metastasis. In addition, we found that upregulation of SIX4 promoted HCC invasion and metastasis and knockdown of SIX4 expression decreased HCC invasion and metastasis. These studies suggested that SIX4 was a prognostic biomarker of HCC. The first step of cancer metastasis is growth of neoplastic cells [31]. Primary tumor grows need to develop a blood supply for metabolic needs, which is called angiogenesis. This process can also provide an escape route by which cells can leave the tumor and enter into the body's circulatory blood system. Once the metastatic cells in the new site, cells must initiate and maintain growth for a macroscopic tumor to form [32, 33]. In this work, we found that upregulation of SIX4 promoted HCC proliferation and knockdown of SIX4 expression decreased HCC proliferation. Therefore, SIX4-mediated HCC proliferation may be a reason for SIX4-mediated HCC metastasis.

YAP1 is essential for cancer initiation, progression, and metastasis in several solid tumors through interacting with TAZ and other transcription factors [34]. YAP1 is an independent prognostic marker in HCC and promote liver tumorigenesis through the cooperation between MYC and β-catenin [35, 36]. Liver-specific YAP1 overexpression leads to liver tumor in transgenic mice [37]. YAP1 overexpression promotes HCC progression and metastasis




through enhancing the expression of genes involved in proliferation and stemness and regulating metabolism and cytoskeleton [24, 38, 39]. c-MET is a well-known oncogene in human cancers and is required to maintain the transformed and metastatic phenotype [40, 41]. c-MET aberrations occur in nearly half of HCC patients through several

◀ **Fig. 5 SIX4 is essential for HGF-induced HCC metastasis.** **a** Western blotting was used to detect the SIX4 expression in PLC/PRF/5 cells treated with HGF or combined HGF treatment and SIX4 knockdown. **b** Transwell shown the migratory and invasive abilities of PLC/PRF/5 cells treated with HGF or combined HGF treatment and SIX4 knockdown. **c** Western blotting analyzed the HGF and SIX4 expression in PLC/PRF/5 cells transfected with LV-shcontrol or LV-HGF lentivirus and in PLC/PRF/5-HGF cells transfected with LV-shcontrol or LV-shSIX4 lentivirus. **d** Transwell shown the migratory and invasive abilities of PLC/PRF/5-HGF cells transfected with LV-shcontrol or LV-shSIX4 lentivirus. **e–i** In vivo assays shown that SIX4 knockdown can inhibit HGF-induced HCC metastasis. **e** The nude mice were injected with the indicated cells in the liver. Representative Bioluminescence images were shown in different groups. **f** Incidence of lung metastasis in the nude mice. **g** The number of metastatic lung nodules in lung. **h** Overall survival time of nude mice in different groups. **i** Representative H&E staining images of lung tissues from nude mice in different groups. $*P < 0.05$.

ways and is identified as an oncogenic driver in HCC [19, 20]. Therefore, c-MET is a promising target in HCC [23]. These evidences indicate the crucial roles of both YAP1 and c-MET in promoting HCC progression. In this study, we illustrated that SIX4 upregulated YAP1 and c-MET expression through directly binding to their promoters. Knockdown of YAP1 and c-MET decreased SIX4-enhanced HCC metastasis, whereas ectopic overexpression of YAP1 and c-MET rescued the decreased HCC metastasis induced by SIX4 knockdown. In human HCC samples, SIX4 expression was positively correlated with YAP1 and c-MET expression, and patients with positive coexpression of SIX4/ nuclear YAP1 or SIX4/c-MET exhibited the worst prognosis. Taken together, both clinical evidence and experimental data demonstrated that SIX4 promoted HCC metastasis through upregulating YAP1 and c-MET expression.

The regulatory mechanism of SIX4 overexpression in human HCC remains unclear. SIX1 is upregulated in Ink4a/Arf-deficient mice transgenic for hepatocyte growth factor/scatter factor (HGF/SF) and has been proved to be an important factor in promoting metastasis of rhabdomyosarcoma (RMS) [42]. This study suggested that HGF may regulate other SIX family members. Therefore, HGF, the specific ligand of c-MET, attracted our attention. Physiologically, HGF-c-MET signaling participates in embryogenesis, wound healing, organ regeneration, angiogenesis and morphogenic differentiation [18, 43]. Moreover, HGF-c-MET signaling plays a critical role in promoting HCC angiogenesis, invasion, and metastasis [40, 41, 44, 45]. The elevated expression of both HGF and c-MET are associated with poor prognosis in human HCC patients [21, 46, 47]. In this study, we found that HGF-c-MET signaling upregulated SIX4 expression through the ERK/NF-kB pathway. SIX4 upregulated the receptor c-MET expression, which formed an HGF-SIX4-c-MET positive feedback loop. Furthermore, knockdown of SIX4 dramatically decreased HGF-mediated HCC metastasis. Thus, we defined an HGF-SIX4-c-MET positive feedback loop that played a pivotal role in HCC metastasis, and targeting this pathway may provide new therapeutic strategy to inhibit HCC metastasis.

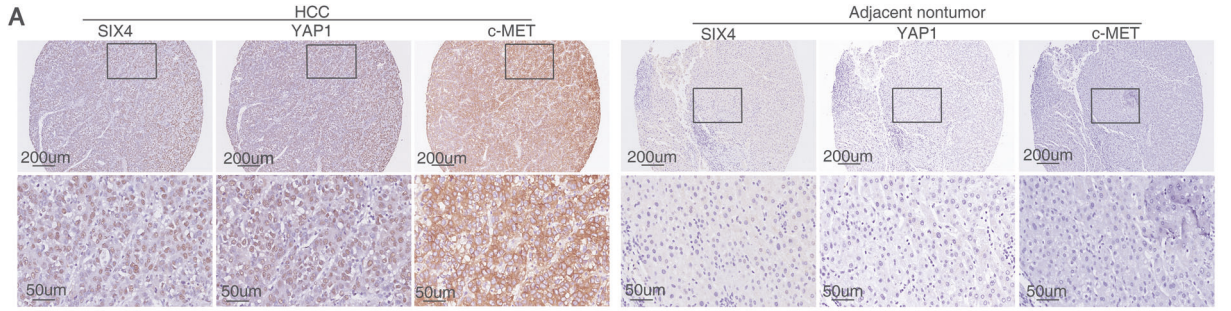
To design pharmacological strategy against this positive feedback loop, we focused on both YAP1 and c-MET inhibitors. c-MET inhibitor has been proved to inhibit tumor growth in mice model [48]. However, clinical trials of c-MET inhibitor treatment alone have failed to produce satisfactory outcomes in HCC [20, 23, 30]. Combined treatment of c-MET and EGFR inhibitors in therapy of colorectal cancer, non-small cell lung cancer and HCC have been processed [49–51]. Verteporfin is a YAP1 inhibitor through disrupting the formation of the YAP1-TEAD complex [29]. Verteporfin inhibits liver cancer initiation and enhances the effect of sorafenib in HCC [52, 53]. Based on these studies, we hypothesized that whether the combination of YAP1 inhibitor Verteporfin and c-MET inhibitor Capmatinib had any effect on HCC metastasis. Our in vivo data showed that the combination of both inhibitors dramatically suppressed SIX4-mediated HCC metastasis compared with control or single agent alone. These results provided a new therapeutic strategy to inhibit SIX4-driven HCC metastasis.

In conclusion, we demonstrated that upregulated SIX4 induced by HGF promoted HCC metastasis through transactivating YAP1 and c-MET expression. The combination treatment of YAP1 and c-MET inhibitors significantly suppressed SIX4-mediated HCC metastasis. Thus, SIX4 is a prognostic biomarker in HCC patients and targeting the HGF-SIX4-c-MET positive feedback loop may provide a promising strategy for the treatment of SIX4-driven HCC metastasis.

## Materials and methods

### Cell culture

Immortalized liver cell lines (HL-7702) and HCC cells (Huh6 and JHH-7) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science, China. Human HCC cells (HepG2, Huh-7, Hep3B, PLC/PRF/5, SNU423, SNU398, and SNU449) were purchased from the American Type Culture Collection. Additional human HCC cells (MHCC97H, HCCLM3, and HCCLM6) were kindly provided by Dr Tang ZY (Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). MHCC97H, HCCLM3, and HCCLM6 cells are stepwise metastatic potential cell lines with the same genetic background but different lung metastatic potentials. HepG2, Hep3B, Huh7, and PLC/PRF/5 are HCC cells with low metastatic potential, whereas MHCC97H, HCCLM3, and HCCLM6 are HCC cells with high metastatic potential. Cells were cultured in Dulbecco's Modified




B

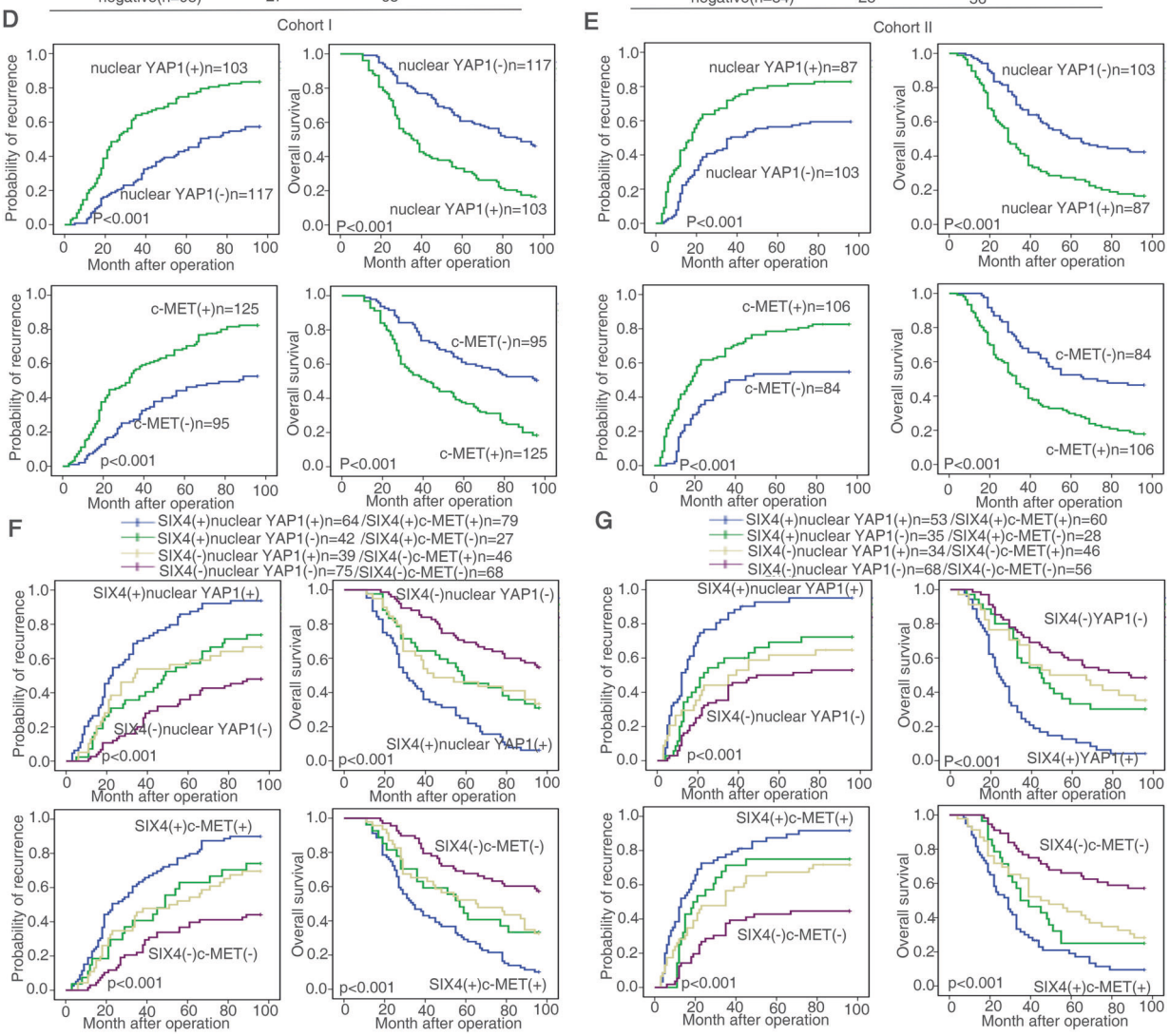
Correlation analysis of SIX4 and nuclear YAP1 or c-MET expression in cohort I (220) HCC tissues

| | | SIX4 positive(n=106) | SIX4 negetive(n=114) | P value |
|---|---|---|---|---|
| nuclear YAP1 | positive(n=103) | 64 | 39 | <0.001 |
| | negative(n=117) | 42 | 75 | |
| c-MET | positive(n=125) | 79 | 46 | <0.001 |
| | negative(n=95) | 27 | 68 | |

C

Correlation analysis of SIX4 and nuclear YAP1 or c-MET expression in cohort II (190) HCC tissues

| | | SIX4 positive(n=88) | SIX4 negative(n=102) | P value |
|---|---|---|---|---|
| nuclear YAP1 | positive(n=87) | 53 | 34 | <0.001 |
| | negative(n=103) | 35 | 68 | |
| c-MET | positive(n=106) | 60 | 46 | 0.002 |
| | negative(n=84) | 28 | 56 | |




Eagle Medium (DMEM) at 37 °C in a 5% CO2 incubator. The medium was supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. These above cell lines were authenticated by short tandem repeats (STRs) DNA profiling. All cells were tested for mycoplasma contamination before use with the Universal Mycoplasma

◀ **Fig. 6 SIX4 expression is positively correlated with nuclear YAP1 and c-MET expression in human HCC tissues. a** Representative IHC staining images shown SIX4, nuclear YAP1 and c-MET expression in human HCC tissues. **b**, **c** The correlation analysis of the expression of SIX4 and nuclear YAP1 or SIX4 and c-MET in human HCC tissues from cohort I (**b**) and cohort II (**c**). **d**, **e** Overall survival time and recurrence in HCC patients with positive or negative expression of nuclear YAP1 or c-MET in cohort I (**d**) and cohort II (**e**) were shown. **f**, **g** Kaplan–Meier analyzed recurrence and overall survival times of patients with coexpression of SIX4/ nuclear YAP1 or SIX4/c-MET in cohort I (**f**) and cohort II (**g**).

Detection Kit (ATCC 30–1012 K) and were not contaminated by mycoplasma.

## Patients and follow-up

This study was approved by the Ethics Committee of Tongji Medical College. All patients provided full consent for the study. Cohort I included 220 adult patients with HCC who underwent curative resection between 2003 and 2005 at the Tongji Hospital of Tongji Medical College (Wuhan, China). Cohort II included 190 adult patients with HCC who underwent curative resection between 2006 and 2008 at the Tongji Hospital of Tongji Medical College (Wuhan, China). A preoperative clinical diagnosis of HCC was based on the diagnostic criteria of the American Association for the Study of Liver Diseases. The inclusion criteria were as follows: (a) distinctive pathologic diagnosis; (b) no preoperative anticancer treatment or distant metastases; (c) curative liver resection; and (d) complete clinicopathologic and follow-up data. The differentiation statuses were graded according to the method of Edmondson and Steine. The pTNM classification for HCC was based on The American Joint Committee on Cancer/International Union Against Cancer staging system (6th edition, 2002). Follow-up data were summarized at the end of December 2013 (Cohort I) and December 2016 (Cohort II, range 4–96 months) respectively. The patients were evaluated every 2–3 months during the first 2 years and every 3–6 months thereafter. All follow-up examinations were performed by physicians who were blinded to the study. During each check-up, the patients were monitored for tumor recurrence by measuring the serum AFP levels and by performing abdominal ultrasound examinations. A computed tomography and/or magnetic resonance imaging examination was performed every 3–6 months, together with a chest radiographic examination. The diagnostic criteria for HCC recurrence were the same as the preoperative criteria. The time to recurrence and overall survival were the primary endpoints. The time to recurrence was calculated from the date of resection to the date of a diagnosis with tumor recurrence. The overall survival was calculated from the date of resection to the date of death or of the last follow-up.

In addition, 10 normal liver tissues, 50 pairs of fresh HCC tissues and adjacent nontumor tissue samples and 30 pairs of fresh metastatic and matched primary HCC tissues and adjacent nontumor tissue samples were collected after surgical resection and were used to investigate the mRNA expression levels of SIX4.

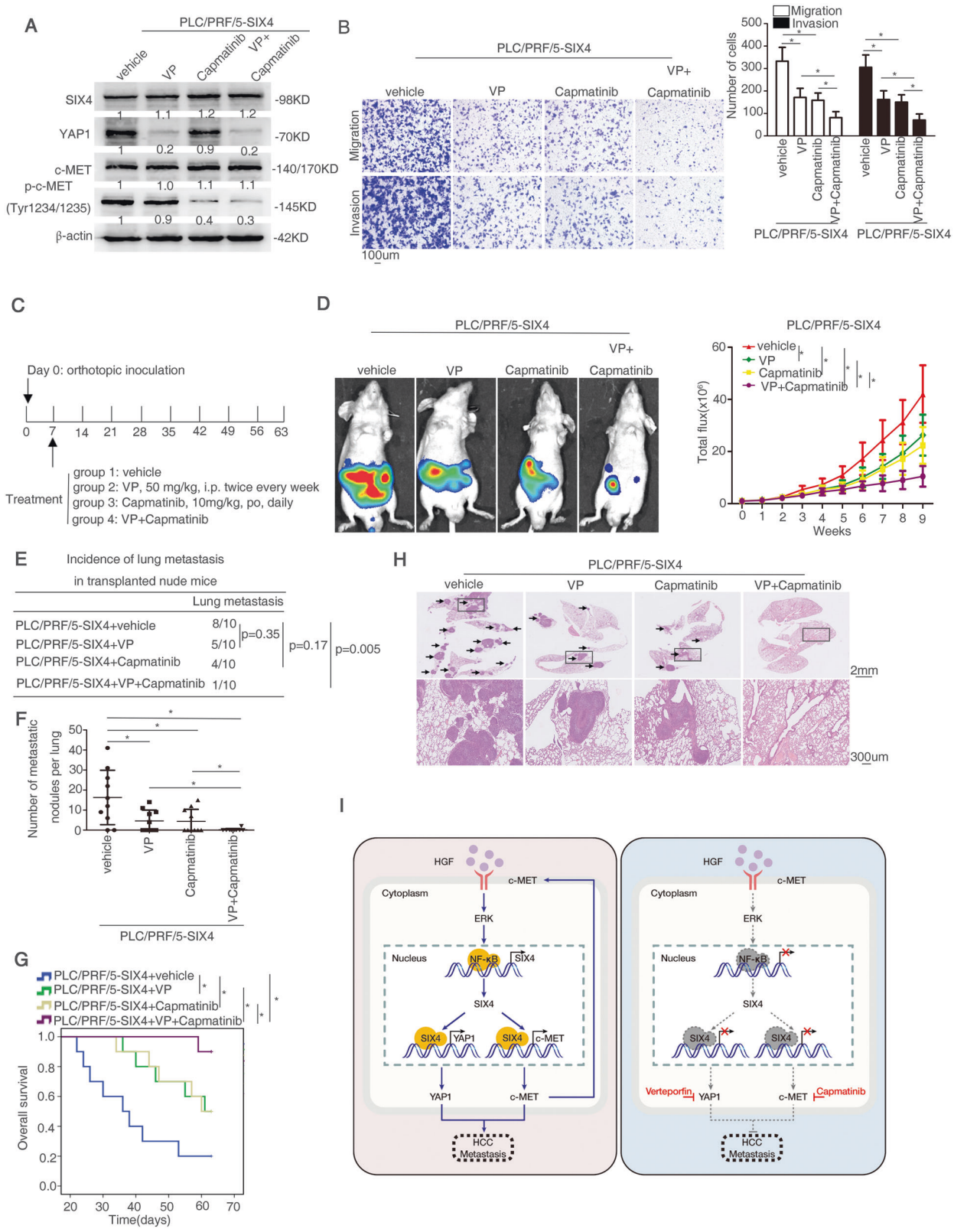
## Construction of tissue microarrays and immunohistochemistry

HCC samples and the corresponding adjacent liver tissues were used to construct a tissue microarray (Shanghai Biochip Co., Ltd. Shanghai, China). IHC was performed on 4 μm-thick, routinely processed paraffin-embedded sections. Briefly, the tissue sections were deparaffinized after baking at 60 °C for an hour. Endogenous peroxidase activity was blocked by 3% (vol/vol) hydrogen peroxide in methanol for 12 min and washes with phosphate-buffered saline (PBS). Then the slides were immersed in 0.01 mol/L citrate buffer solution (pH 6.0) and placed in a microwave oven for 30 min. After washed with PBS, the sections were incubated with the primary antibody diluted in PBS containing 1% (wt/vol) bovine serum albumin in 4 °C for overnight. The tissue microarray was stained for SIX4 (MilliporeSigma, SAB1405112), YAP1 (Cell signaling technology, #14074), c-MET (Cell signaling technology, #8198), HGF (Abcam, ab83760), HGF (Cell signaling technology, # 52445) and KI67 (Abcam, ab15580) expression. Negative controls were performed by replacing the primary antibody with preimmune mouse serum. After washed with PBS, the sections were treated with a peroxidase-conjugated second antibody (Santa Cruz) for 30 min at room temperature and then washed with PBS. Reaction product was visualized with diaminobenzidine for 2 min. Images were obtained under a light microscope (Olympus, Japan) equipped with a DP70 digital camera.

Analyses were performed by two independent observers who were blinded to the clinical outcome. The immunostaining intensity was scored on a scale of 0–3: 0 (negative), 1 (weak), 2 (medium) or 3 (strong). The percentage of positive cells was evaluated on a scale of 0–4: 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The final immunoactivity scores were calculated by multiplying the above two scores, resulting an overall score which range from 0 to 12. Each case was ultimately considered "negative" if the final score ranges from 0 to 3, and "positive" if the final score ranges from 4 to 12 as described previously [54].

## In vivo metastatic model and bioluminescent imaging

All animal experiments were approved by the Committee on the Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. BALB/C nude




mice (male, 5 weeks old) were housed and cared according to the institutional guidelines for animal care. Mice were randomly assigned into experimental or control groups, blinding was not possible. For in vivo metastasis assay, $2 \times 10^6$ cells were resuspended in 50 μL PBS/matrigel mixture. Under anesthesia, mice were orthotopically inoculated in

◀ **Fig. 7 Combined treatment of YAP1 and c-MET inhibitor blocks SIX4-medicated HCC Metastasis. a** PLC/PRF/5-SIX4 cells treated with YAP1 inhibitor VP or c-MET inhibitor Capmatinib alone, or the combination of both inhibitors. Western blotting shown the expression of SIX4, YAP, and c-MET. **b** Transwell shown the migratory and invasive abilities of PLC/PRF/5-SIX4 cells in different treatment groups. **c** The diagram of vivo treatment in nude mice. One week after injection of PLC-PRF/5-SIX4 cells, these mice were randomly divided into four group and treated with vehicle, Verteporfin or Capmatinib or combined treatment. **d–h** In vivo assays shown that combined treatment of YAP1 and c-MET inhibitor can largely block HCC metastasis. **d** Representative Bioluminescence images were shown in different groups. **e** Incidence of lung metastasis in the nude mice. **f** The number of metastatic lung nodules in lung. **g** Overall survival time of nude mice in different groups. **h** Representative H&E staining images of lung tissues from nude mice in different groups. **i** A schematic diagram of the role of HGF-SIX4 signaling in HCC metastasis. HGF-c-MET signaling upregulates SIX4 expression through ERK/NF-kB pathway. SIX4 promotes HCC metastasis through transactivating YAP1 and c-MET. Combined YAP1 inhibitor Verteporfin and c-MET inhibitor Capmatinib almost abolished SIX4-induced HCC metastasis. $^{*}P < 0.05$.

the left hepatic lobe with the indicated cells through an 8 mm transverse incision in the upper abdomen ($n = 10$ mice/group). For drug-based drug intervention, mice were given daily oral doses of Capmatinib 10 mg/kg [30] and/or intraperitoneal injection of Verteporfin (VP) 50 mg/kg [52] twice every week. The in vivo tumor formation and metastases were monitored using the bioluminescence. For in vivo signal detection, D-luciferin (Perkin-Elmer) at 100 mg/kg was injected intraperitonially into the nude mice. Bioluminescent images were captured using a Lago X optical imaging system Imaging System (SI Imaging). At the 9 weeks, the mice were sacrificed and the lungs were collected for histological examination.

## Statistical analysis

All values were recorded as the mean ± standard deviation (sd). $P$ values were statistically analyzed by the $\chi 2$ test for categorical variables and by Student's test for quantitative data. Survival was calculated with the Kaplan–Meier method (log-rank test). Multivariate analysis was performed by Cox regression analysis. $P < 0.05$ was considered statistically significant. Statistical values were calculated with SPSS software (Version 20.0).

All other materials and methods can be found in the supplementary materials.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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